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Applicant: Stephen James Brocchini et al

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For: Conjugated Biological Molecules and Their Preparation

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. § 1.132

Sir:

I, Andrew John Timothy George, hereby declare that:

1. My name is Andrew John Timothy George, of Imperial College, London. I have been Professor of Immunology in the Division of Medicine since 2002. I am also an Honorary Professor in the Institute of Ophthalmology in University College London, and in 2005 was visiting Professor at Flinders University, Adelaide and the John Radcliffe Hospital in Oxford. I took my BA in Natural Sciences in 1984 at the University of Cambridge, and my PhD in immunochemistry at the University of Southampton in 1987. I am a Fellow of the Royal College of Pathologists. Until 2002 I was course organiser of the MSc in Immunology at which time I was awarded a BBSRC Research Development Fellowship to concentrate on my research. I have twice been given an award for excellence in teaching by Imperial College. I run a research team developing molecular therapies for a range of conditions, and have more than 170 papers published or in press. I am named as inventor on a number of patent

applications, and have co-edited a book entitled "Diagnostic and Therapeutic Antibodies". I have acted as an expert witness in a number of court cases.

2. I have known Professor Sunil Shaunak for a number of years. In November 2005, Prof. Shaunak came to my office to talk to me about some work he had been doing. I cannot remember the exact words which were used in the conversation, but he told me that he and colleagues had developed a process for conjugating PEG to proteins which involved breaking a sulfur-sulfur bond in the protein. The early work had been carried out using interferon. He told me that the resulting PEG-interferon conjugate retained virtually the full activity of the native interferon. I told him that I was very surprised to hear this, as I would have expected a very significant reduction in activity. He told me that he was intending to publish a paper in Nature, and I asked him to send me a copy of his paper. This paper was subsequently published online by Nature Chemical Biology in April 2006, and a further article was published in the May 2006 edition of Hospital Doctor. Copies of these papers are attached to this Declaration.

3. Subsequent to my conversation with Prof. Shaunak, I was shown a copy of the original PEGylation experiments, as they appear in a patent application filed by Prof. Shaunak and colleagues. These experiments confirm what I was told by Prof. Shaunak, i.e. that the PEG-interferon conjugate retained virtually the full activity of the native interferon. I remain surprised that that you can replace the disulfide bond in interferon with a cross linking agent that added PEG onto the molecule - not because the chemistry would be difficult, but because I assumed that disrupting the disulfide bond in this way would alter the properties of the molecule. The fact that disrupting disulfide bonds alters the properties, and particularly the biological properties, of the protein, is extremely well known, and prior to speaking with Prof. Shaunak, I would not have thought that such an approach to PEGylation was worth trying. I would have expected the resultant PEGylated protein to lack the desired biological activity.

4. I declare that all statements made herein of my own knowledge and belief are true and that all statements made on information and belief are believed to be true, and further, that the statements are made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that

such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 12 MARCH 2009

Andrew George, MA PhD FRCPath

Attachment A

nature
chemical biology

Site-specific PEGylation of native disulfide bonds in therapeutic proteins

Sunil Shaunak[1], Antony Godwin[2], Ji-Won Choi[1], Sibu Balan[2], Elisa Pedone[2], Damotharan Vijayarangam[1], Sibylle Heidelberger[1], Ian Teo[1], Mire Zloh[3] & Steve Brocchini[2]

Native disulfide bonds in therapeutic proteins are crucial for tertiary structure and biological activity and are therefore considered unsuitable for chemical modification[1,2]. We show that native disulfides in human interferon α-2b and in a fragment of an antibody to CD4[1] can be modified by site-specific bisalkylation of the two cysteine sulfur atoms to form a three-carbon PEGylated bridge. The yield of PEGylated protein is high, and tertiary structure and biological activity are retained.

It is generally considered that a protein's native disulfide bonds cannot be modified because they are crucial to its structure and function[1,2]. Covalent conjugation of poly(ethylene glycol) (PEG) to therapeutic proteins increases their *in vivo* stability by protecting the protein from degradation, masking its immunogenic sites and reducing clearance[3]. Typically, PEGylation uses nonspecific reactions with nucleophilic residues and produces mixtures of PEGylated positional isomers[4]. To solve this problem, we explored the reactivity of the two sulfur atoms of a native disulfide for selective conjugation of PEG using a thiol-specific, cross-functionalized PEG monosulfone (Fig. 1a). Mechanistically, the conjugated disulfide bond in the PEG monosulfone is necessary to initiate a sequence of addition-elimination reactions[5,6]. After addition of thiol, elimination of sulfinic acid generates another conjugated double bond for the second thiol (**Supplementary Scheme 1** and **Supplementary Methods** online). This leads to the formation of a three-carbon bridge between two sulfur atoms. Disulfide-scrambling reactions are inhibited because of thiol propinquity in the nondenatured protein and by having the bisalkylation functionality at the end of PEG.

We used interferon α-2b (IFN) because it is representative of four-helical-bundle proteins with accessible disulfide bonds. Theoretically, the effect of introducing a three-carbon bridge is determined using stochastic dynamics simulations. The bridged IFN isomers Cys1-CCC-Cys98 and Cys29-CCC-Cys138 are within the conformational flexibility of the crystal and NMR-based structures of interferon α-2a, indicating that IFN's tertiary structure is preserved[7] (**Supplementary Results** 1 online).

We found that a three-carbon disulfide-bridged PEG-IFN can be prepared when one protein equivalent (equiv.) of PEG monosulfone is used after reducing both disulfides. Conjugation is conducted at pH 7.8 and 4 °C for 2 h after removal of excess dithiothreitol. If two equivalents of PEG monosulfone are used, both disulfides undergo conjugation. As a control, we conjugated a non-PEG precursor to IFN. SDS-PAGE gels showed IFN's conjugation to precursor and PEG monosulfone, with MALDI-TOF–MS confirming the M_w of the isomers Cys1-CCC-Cys98 and Cys29-CCC-Cys138 (Fig. 1b–d) and of their trypsin-digested fragments (**Supplementary Results** 2 online). The three-carbon–bridged PEG-IFNs were purified by

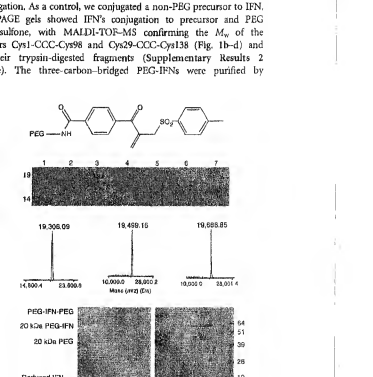

Figure 1 Structural characterization. (a) PEG monosulfone. (b) Silver-stained gel of the non-PEGylated three-carbon (190 Da) disulfide-bridged IFN. Lanes: (1) M_w markers (kDa); (2) IFN; (3) reduced IFN; (4) 1 equiv. bisulfone showing IFN (upper), single-bridged (middle) and double-bridged (lower) IFN; (5 and 6) 2 and 4 equiv., respectively, showing single-bridged (upper) and double-bridged (lower) IFN; (7) 6 equiv. showing double-bridged IFN. (c) MALDI-TOF–MS of IFN (left), Cys-CCC-Cys IFN (middle) and double-bridged IFN (right). (d) Gels stained with colloidal blue (protein) and barium iodide (PEG, right). Lanes: (1) 20 kDa PEG; (2) IFN; (3) IFN with reduced disulfide; (4) PEGylation reaction mixture; (5) IFN with both disulfides reduced.

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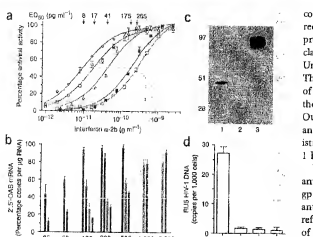

Figure 2 Biological activities. (a) Antiviral activity in A549 cells infected with EMC virus ($n = 6$). (b) 2′,5′-OAS mRNA synthesis in Molt-4 cells ($n = 3$). IFN (gray); unreacted IFN recovered after SEC-HPLC (red); non-PEGylated three-carbon disulfide-bridged IFN (green); three-carbon disulfide single-bridged 10 kDa PEG-IFN (orange); three-carbon disulfide single-bridged 20 kDa PEG-IFN (blue). (c) Immunoblot with an antibody to Fab. M_r markers (left) are in kDa. Lanes: (1) Fab, (2) reduced Fab, (3) three-carbon disulfide single-bridged 20 kDa PEG-Fab. (d) Inhibition of HIV-1 entry into human C8166 (T-lymphoblastoid) cells as determined by real-time PCR for RU5, the first DNA transcript of HIV-1 to be synthesized after viral entry ($n = 3$). Data presented as mean ± s.e.m.

cation-exchange chromatography followed by size-exclusion chromatography (SEC)-HPLC with confirmation by western immunoblotting. The SEC-HPLC chromatogram showed a three-carbon disulfide single-bridged PEG-IFN (that is, Cys1-CC[PEG]C-Cys98 or Cys29-CC[PEG]C-Cys138, yield ≤5%), a three-carbon disulfide double-bridged PEG-IFN (Cys1-CC[PEG]C-Cys98 and Cys29-CC[PEG]C-Cys138, yield 23.5%), IFN (yield 4.9%) and aggregated IFN (yield 6.6%) (**Supplementary Results 3 online**).

The reaction can be simplified *by in situ* conversion of the PEG bisulfone to the PEG monosulfone at pH 7.8 during protein conjugation. Competitive reactions of the PEG monosulfone with other nucleophilic residues are not seen (**Supplementary Results 4 online**). MALDI-TOF-MS confirmed the M_w of the two-bridged PEG-IFN isomers, and CD confirmed the preservation of IFN's α-helical structure (**Supplementary Results 2**).

Interferon α-2b has distinct effects *in vitro*: it blocks infection of human A549 (lung epithelial) cells by encephalomyocarditis (EMC) virus, it induces 2′,5′-oligoadenylate synthetase (2′,5′-OAS) mRNA synthesis, and it upregulates major histocompatibility (MHC) class I expression on immunoregulatory cells (**Supplementary Methods**). Using SEC-HPLC, we found that the unreacted IFN and the non-PEGylated three-carbon disulfide single-bridged IFN both showed a small reduction in antiviral activity compared to IFN (**Fig. 2a,b**). Our results also showed that insertion of a three-carbon disulfide bridge contributed ~11%, and addition of PEG contributed ~89% to the reduction in the PEG-IFN's biological activity. Because PEG reduces protein immunogenicity, the PEG-IFNs have a lower affinity for MHC class I molecules than IFN (**Supplementary Results 5 online**). Uniquely, the PEG's length does not affect its biological activities. The PEG-IFN's biological activities (~8% of IFN) are similar to those of the PEG-IFN in clinical use (~7%)[8–10], the enhanced *in vivo* therapeutic efficacy compensating for the reduced *in vitro* activity[10]. Our PEG-IFNs are stable in aqueous solution for 3 months at 4 °C, and in human serum for 30 h at 37 °C. After subcutaneous administration in mice, the 20 kDa PEG-IFN's half-life is 12 h compared to 1 h for IFN.

We applied this approach to a human CD4 receptor-blocking antibody fragment (Fab). Entry of HIV-1 into cells requires viral gp120 to bind the D1 domain of human CD4. The IgG_1 monoclonal antibody Q4120/ADP318 (which binds the D1 domain of CD4; **ref. 11**) was digested to make Fab and PEGylated after reduction of its interchain disulfide (**Fig. 2c**). At a saturating dose, the PEG-Fab was as effective as Fab at blocking HIV-1 entry into $CD4^+$ T-lymphocyte cells (**Fig. 2d**).

Our studies also include the PEGylation of L-asparaginase without loss of enzyme activity or immunogenicity[12]. The accessible native disulfide bonds of proteins can therefore be modified by the site-specific insertion of a three-carbon PEGylated bridge. Our approach differs fundamentally from conjugation of PEG to amine residues[8–10], where the biological activity of the PEGylated positional isomers depends upon conjugation conditions and the size of PEG[4]. It also makes engineering free cysteines into proteins for thiol-selective PEGylation unnecessary. As the biological activities of our PEGylated proteins are independent of PEG size, only their *in vivo* pharmacokinetics need optimizing before clinical trials.

Note: Supplementary information is available on the Nature Chemical Biology website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Chemical Biology* website for details).

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Attachment P

Sick of poverty: the world's poorer nations have little prospect of affording the drugs their populations need at current commercial prices

'People in academic medicine have a choice. They can use their ideas and creativity to make large sums of money for small numbers of people, or they can look outwards to the global community and make affordable treatments for common diseases'
Prof Sunil Shaunak

When cash is no objective

At a time when even the NHS can't afford the latest drugs, is there any hope for the world's poor countries? Yes, there is – as Janis Smy finds out from an altruistic London-based doctor who is developing a treatment for hepatitis C

When satirist Tom Lehrer lampooned doctors who 'specialise in diseases of the rich', he clearly did not have Prof Sunil Shaunak in mind.

The London-based clinician and academic is powerfully motivated by the global burden of preventable and treatable disease, and is determined to find ways of providing medicines that the poorest people in the world can afford.

At a time when the NHS has to question the use of expensive treatments such as Herceptin and inhaled insulin, the benefits of altruistic research may also extend to those of us in the developed countries.

Prof Shaunak, consultant physician at Hammersmith and Chelsea and Westminster hospitals and professor of infectious diseases at Imperial College, has his sights fixed on hepatitis C, which infects more than 170 million people worldwide, causing a huge burden of chronic liver disease and premature death.

He stands prepared to challenge long-held principles of protein chemistry, to pit his wits against the pharmaceutical giants and to parley with governments.

Hepatitis C is optimally managed by a combination of the broad-spectrum antiviral ribavirin and a form of the immunomodulatory protein interferon-alpha, chemically modified to extend its half-life. The all-important modification involves attaching polyethylene glycol (PEG) polymers to the otherwise relatively small immune protein, making it large enough to withstand rapid metabolism and excretion.

The process, known as pegylation, has proven to be a money-spinner for the pharmaceutical giants, which command high prices for their treatments. Pharmacists at the Hammersmith report that a course of combined hepatitis C therapy for one patient costs about £7,000.

Now Prof Shaunak, in collaboration with Prof Steve Brocchini, a research chemist at the London School of Pharmacy, has developed a new method of pegylation which does not infringe existing patents. The resulting molecule, recently reported in *Nature*, appears to be as effective as the existing product.

Unlike their commercial rivals, however, the collaborators have no intention of growing rich from their discovery.

'People in academic medicine have a choice,' says Prof Shaunak. 'They can use their ideas and creativity to make large sums of money for small numbers of people, or they can look outwards to the global community and make affordable treatments for common diseases.'

The new combined treatment for hepatitis C, using the alternative pegylated interferon, enters fast-track clinical trials in India next year, funded by the Indian government.

Sir Michael Arthur, British High Commissioner to India, applauds the plan. 'The technology transfer agreement is a shining example of how exciting innovations in our best universities can be rapidly turned into new and useful healthcare products,' he says.

Shantha, a pharmaceutical company in Hyderabad, has been granted use of the technology in view of its record in manufacturing affordable healthcare products yet still making enough profit to stay in business. Its version of hepatitis B vaccine costs about US$1.25 per course, compared with about $125 charged by the multinationals. It is widely used by developing countries and has been adopted by the World Health Organization.

For Prof Shaunak, the development of the new pegylated interferon alpha molecule is the culmination of a career spent challenging accepted mores. And it's an ethos that began when he was only a junior.

He says: 'When I was a trainee, I was astonished to see how doctors sat in little boxes. The best research was being carried out in these territorial enclaves. I wanted to stay outside of the boxes, and look at medicine beyond any individual organ-based system – hence my commitment to infectious diseases.'

His chosen field did not seem to offer the most promising career – infectious diseases were considered to be pretty much conquered. Then AIDS rocked the world, involving not just an initial organism, but also showing how large numbers of immunosuppressed patients could be hit simultaneously by multiple pathogens, opening up the concept of multi-drug therapy for infectious disease.

Prof Shaunak moved to the US to specialise in HIV. 'In the UK,' he says, 'HIV is mainly the province of genito-urinary doctors but in America it is looked at by infectious disease specialists as part of internal medicine.'

The experience opened his eyes to the yawning healthcare divide between rich and poor – and stirred him to find ways to bridge it.

'HIV patients who couldn't pay got no medicines,' he says. 'So if one of our more affluent patients died, we asked the family to return unused HIV drugs, which we reissued to those who would otherwise have no treatment at all. It was breaking all the rules.'

But breaking the rules has become a stock in trade for Prof Shaunak. He cheerfully admits that the crucial step in the new pegylation process would never have been developed had he and Prof Brocchini understood and respected one of the central tenets of protein chemistry.

Previous forms of pegylation involved attaching numerous PEGs to the outside of the interferon, comparable to bubble-wrapping. Prof Shaunak and Prof Brocchini circumvented the existing patents by breaking a disulphide bond in the protein, creating a bridge and using that as an attachment point for PEG.

'Later,' he says, 'we learnt that the disulphide bonds should never be interfered with if you hope to retain biological function. Fortunately, we hadn't read the protein text books.'

The collaborators are confident that disulphide bond-based pegylation can be used to make affordable versions of other therapeutically useful biological proteins.

Prof Shaunak believes the work will form part of the 'revolution' he believes is about to hit the research environment.

'The pharmaceutical industries haven't done anything to help a large proportion of people around the world,' he says. 'But we live in a global community. The idea that we can ignore what happens in the developing world no longer applies. People already realise that diseases such as bird flu and severe acute respiratory syndrome (SARS), which begin thousands of miles away, can have a big effect on us. We need global solutions to these global challenges.'

He concludes: 'The Make Poverty History campaign is an example of what people can do when they are determined. I hope young doctors now in training will see just how exciting work like this can be.'

Prof Shaunak's molecule is an alternatively pegylated interferon that crucially breaks a disulphide bond to create an attachment point for the PEG polymer